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Growth inhibition of malignant glioblastoma by DING protein

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Abstract

Malignant gliomas are a highly aggressive type of brain tumor with extremely poor prognosis. These tumors are highly invasive and are often surgically incurable and resistant to chemotherapeutics and radiotherapy. Thus, novel therapies that target pathways involved in growth and survival of the tumor cells are required for the treatment of this class of brain tumors. Previous studies revealed that epidermal growth factor receptor and extracellular-signal-regulated kinases (ERKs), which are involved in the induction of cell proliferation, are activated in the most aggressive type of glioma, i.e. glioblastoma multiforme (GBM). In fact, GBMs with increased levels of ERK activity exhibit a more aggressive phenotype than the others with moderate ERK activity, pointing to the importance of ERK and its kinase activity in the development and progression of these tumors. In this study, we have evaluated the effect of p38SJ, a novel member of the DING family of proteins, derived from *Hypericum perforatum* calluses, on the growth of

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malignant glioma cell lines, T98G and U-87MG by focusing on cell cycle and signaling pathways controlled by phosphorylation of various regulatory proteins including ERK. p38SJ, which exhibits profound phosphatase activity, shows the capacity to affect the phosphorylation status of several important kinases modulating signaling pathways, and cell growth and proliferation. Our results demonstrate that p38SJ reduces glioma cell viability and arrests cell cycle progression at G0/G1. The observed growth inhibitory effect of p38SJ is likely mediated by the downregulation of several cell cycle gatekeeper proteins, including cyclin E, Cdc2, and E2F-1. These results suggest that p38SJ may serve as a potential candidate for development of a therapeutic agent for the direct treatment of malignant gliomas and/or as a potential radiosensitizer.

Keywords

p38SJ; DING; Phosphatase; Glioma; Cell cycle

Introduction

Extracts of the *Hypericum perforatum* plant, also known as St. John's Wort, have captured the interest of chemists and biologists for nearly a century [1]. Peptides and polyphenols from this plant represent a series of promising and intriguing therapeutic compounds for a variety of diseases [2–4]. Compounds from *H. perforatum*, including hypericin and hyperforin, have been evaluated against a spectrum of tumor cells, including melanoma, colon cancer, nasopharyngeal carcinoma, epidermoid carcinoma, breast cancer, endometrial carcinoma, bladder cancer, gliomas, and squamous cell carcinoma. Moreover, different agents within the plant have demonstrated effects on free-radical production, angiogenesis and cell signaling [5–14].

Earlier studies in our laboratory led to identification of a novel 27 kDa protein (p27SJ) from extracts of callus cultures of *H. perforatum* with phosphatase activity and the ability to modulate certain cell functions [15]. Further examination of a longer isoform of p27SJ, named p38SJ/DING identified this protein as a member of the DING family of proteins [16, 17]. DING proteins are characterized by an N-terminal Asp-Ile-Asn-Gly-Gly (DINGG) amino acid sequence, and have been shown to exist widely in animals, plants, fungi, eukaryotes, and prokaryotes, though the biochemical properties of these proteins remain largely unclear [18–28]. Previous studies from our laboratory showed the ability of p27SJ to interact physically and/or functionally, with several regulatory proteins including C/EBP β and RNA polymerase II, and modulate expression of viral and cellular genes [16, 17]. Further, overexpression of p27SJ/p38SJ alters cell signaling and proliferation through the hypophosphorylation of ERK1/2 [15]. Studies using the human malignant glioma cell line U-87MG have demonstrated the ability of p27SJ to modestly decrease Cyclin A production via modulation of ERK1/2 and thereby inhibit cell cycle progression at the S and G2 phases of the cell cycle [15].

Here, we demonstrate the capacity of p38SJ in suppressing proliferation of malignant glioma cells, interfering with cell cycle progression and several kinases that control cell proliferation of these tumors.

Materials and methods

Cell culture

U-87MG and T98G human glioblastoma cell lines, and NIH 3T3 mouse fibroblasts were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Cells were maintained in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Life Technologies, Inc.) and antibiotics (100 U/ml penicillin and 10 µg/ml streptomycin) at 37°C in a humidified atmosphere containing 7% CO₂.

Plasmids

The pCDNA6-p27SJ, pCDNA6-p15SJ, pCDNA6-p10SJ, pEYFP-C1 (BD Biosciences Clontech) and pCDNA6 Myc/His (Invitrogen, Carlsbad, CA, USA) plasmids have been described previously [16]. pCDNA6-p38SJ and YFP-p38SJ plasmids have also been described previously [17].

Antibodies

Antibody specific for p38SJ (anti-p27SJ rabbit polyclonal antibody) was generated by Lampire Biological Laboratories, Inc. (Pipersville, PA). Anti-α-tubulin clone B-5-1-2 was obtained from Sigma–Aldrich (Sigma–Aldrich Co., St. Louis, MO). Anti-myc antibody was purchased from Invitrogen (Invitrogen, Carlsbad, CA). Anti-Cdk2 rabbit polyclonal antibody, anti-E2F-1 mouse monoclonal antibody, and anti-Cdk6 monoclonal antibody were purchased from Abcam (Abcam, Cambridge, MA). Anti-cyclin A mouse monoclonal antibody was purchased from Cell Signaling (Cell Signaling Technology, Inc., Danvers, MA). Anti-PCNA rabbit polyclonal antibody, anti-Cyclin E monoclonal mouse antibody, anti-Cdk9 rabbit polyclonal antibody, anti-c-Jun rabbit polyclonal antibody, anti-phospho-c-Jun (KM-1) and anti-Cdc2/p34 mouse monoclonal antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA).

Transfection

T98G and U-87MG cells were transfected with expression plasmids using the Lipofectamine 2000 system (Invitrogen, Carlsbad, CA), per the manufacturers protocol.

Cell viability assay

50×10^3 T98G transfected with either pEYFP-C1 or YFP-p38SJ expressing plasmids were plated into 100 mm dishes. Cells were incubated at 37°C. Each plate was inspected by fluorescent microscopy for expression of YFP and YFP-p38SJ, and the total number of cells counted.

Trypan blue exclusion viability assay

Trypan blue exclusion assay was performed to assess cell viability in T98G cells. 50×10^3 T98G transfected with either pCDNA6 or pCDNA6-p38SJ expressing plasmids were plated into 100 mm dishes. This viability assay measures the percentage of a cell suspension that is able to exclude Trypan blue dye. In brief, cell suspension was diluted 1:1 with 0.4% Trypan blue and cells were counted with a hemocytometer.

Colony formation assay

Approximately 50×10^3 T98G and U-87MG cells were transfected with either pCDNA6, pCDNA6-p38SJ, pEY-FP-C1 or YFP-p38SJ. Cells were incubated at 37°C in blasticidin (5 µg/ml) or 0.8 mM Geneticin (G418) containing media. After 3 weeks, cells were stained with 1% methylene blue for 5 min and the stained colonies in each plate were counted. For each experiment, the counts from triplicate plates were averaged and standard errors calculated.

Proteinase K treatment

p38SJ protein extracts were applied to proteinase K bound to agarose beads (Sigma, St. Louis, MO) in 1.5 ml Eppendorf tubes. The samples were incubated at 37°C with gentle agitation overnight for complete digestion of p38SJ protein. Tubes were then pulse centrifuged at 3,000 rpm and the supernatant extracted and filtered with a 0.2 µm filter. The filtered solution was then incubated at 56°C for 48 h and then inactivated at 95°C for 15 min. Samples were examined by SDS-PAGE followed by staining with Coomassie blue to confirm complete digestion of the p38SJ.

Methylthiazoltetrazolium (MTT) cell proliferation assay

For the MTT assay, we used a cell proliferation kit (MTT) according to the manufacturer's protocol (Roche, Indianapolis, IN, USA). U-87MG or T98G cells were plated onto 96-well plates in triplicate and repeated a minimum of two times at a density of 10,000 cells/well and pre-incubated with either p38SJ protein extract, or with extract after treatment with proteinase K, at 10 µg/ml. After 24 h, 10 µl MTT (5 mg/ml) were added to the wells (final concentration, 0.5 mg/ml) for 4 h, and the reaction was stopped by the addition of 100 µl of solubilization solution. Viable cells with active mitochondria cleave the tetrazolium ring into a visible dark blue formazan reaction product, which was quantified by spectrophotometry in a Dynex MRX Revelation microplate reader (Dynex Technologies, Chantilly, VA) at 570 nm with a reference wavelength of 650 nm. The relative cell viability (percent) was determined as the ratio of average absorbance for treated cells to that for mock, untreated cells.

Fluorescence-activated cell sorting analysis

U-87MG cells were treated with 1 µg/ml of p38SJ protein extract and then harvested at 0, 8, 16, 20, 24, 44 and 52 h. Likewise, NIH 3T3 cells were also treated with 1 µg/ml of p38SJ protein extract and then harvested at 0, 4, 16, 21, 29, 45 and 52 h. Cells were trypsinized and collected in separate 15 ml Falcon tubes. The cells were serially centrifuged and washed in PBS, then resuspended in 1 ml of 70% ethanol at -20°C. Cells were then centrifuged at 5,000 rpm for 5 min and the supernatant was removed. Cells were resuspended in 1 ml of 2.5 µg/ml of propidium iodide and 0.5 mg/ml of RNase A in PBS overnight. Each sample of cells was then analyzed on a Guava Easycyte Mini flow cytometry system (Millipore, Billerica, MA). Data were analyzed using the accompanying Guava Technologies software.

Phospho-MAPK array

A human phospho-MAPK array kit was used from R&D Systems, Inc. (Minneapolis, MN). pEYFP-C1 and YFP-p38SJ transfected T98G cell lysates were applied and processed over the phospho-MAPK arrays as per the manufacturer's protocol.

Protein extract

p38SJ protein extracts were acquired from callus cultures of 1-month old *H. perforatum* plants as described previously [16]. Preparation of protein extracts and immunoblot analysis were done as described previously [16].

Cell treatment and western blot assay

T98G and U-87MG cells were treated with 1 µg of p38SJ extract per ml for 48 h. Cells then were lysed and analyzed by western blot assay [16].

Results

p38SJ inhibits glioblastoma cell growth

To investigate the effect of p38SJ on cell viability of human glioblastoma, plasmids expressing p38SJ alone, pCDNA6-p38SJ, or p38SJ in fusion with the indicator protein, YFP, pYFP-p38SJ, were introduced into T98G cells and their effect on T98G cell viability was examined as described in materials and methods. As seen in Fig. 1a, expression of p38SJ by either plasmid markedly suppressed the growth of T98G cells. Expression of the control plasmid pCDNA6 or plasmids expressing only YFP had no effect on cell viability, indicating that the observed inhibition of cell growth is due to the presence of p38SJ in these cells. A similar response to p38SJ expression was seen in the colony formation assays. In this experiment, pCDNA6-p38SJ or pCDNA6 control plasmid were introduced into T98G and U-87MG cells and the effect of p38SJ on colony formation was examined. A marked decrease in the ability of T98G and U-87MG cells to form colonies was detected upon expression of p38SJ compared to the control cells. Figure 1b (top) illustrates representative data for T98G transfected cells. Quantification of the results showed that ectopic expression of p38SJ caused a 90 or 70% inhibition of colony formation compared to control vector in both, T98G and U-87MG cells, respectively (Fig. 1b, bottom panel). Each experiment was carried out three times.

To determine which region within p38SJ is responsible for suppression of tumor cell growth, we used a collection of mutant variants of p38SJ (shown in Fig. 2a) in colony formation assay. As seen in Fig. 2b, full-length p38SJ showed a robust inhibitory effect on the number of colonies formed on the plate. Removal of the amino acid sequence positioned between 364 and 263 decreased the inhibitory effect of the protein on colony formation and that level remained fairly unchanged upon expression of the smallest mutant protein that encompasses amino acids 1–100. These observations suggest that at least two domains located at the N-terminus between residues 1–100 and the C-terminus, positioned between amino acids 263–364, have a growth inhibitory effect.

In light of earlier reports on the ability of exogenous soluble DING from human T cells to be taken up by the other cells [29], in the next study we investigated the ability of exogenous p38SJ to suppress glioma cell growth. The purity of our p38SJ, which was used in these experiments, was tested by polyacrylamide gel electrophoresis and a single band corresponding to p38SJ was observed (Fig. 3a, lane 1). Treatment of the preparation with proteinase K resulted in complete degradation of p38SJ (Fig. 3a, lane 2). Our results showed that treatment of T98G and U-87MG cells with 10 µg/ml of p38SJ protein had a nearly 50% inhibitory effect on cell growth as measured by MTT assay (Fig. 3b, bars 2 and 4). This effect was reversed upon the treatment of the protein extract with proteinase K indicating that the observed cell growth inhibition of glioblastoma is mediated by p38SJ protein (Fig. 3a, lane 2, and Fig. 3b, bars 3 and 6).

Effect of p38SJ on the cell cycle

In the next series of studies, we asked whether the observed cell growth inhibition by p38SJ is associated with dysregulation of cell cycle, and whether proteins that control cell cycle progression are affected by p38SJ. Results from western blot analyses of protein extract from T98G treated with p38SJ showed a reduced level of expression of proliferating cell nuclear antigen (PCNA) and factors regulating transition between G0/G1 and S phase including Cdk2, cyclin E, Cdc2, p34, and Cdk6 (Fig. 4, Panels a and b). Our results also show a noticeable reduction in Cdk9 as a result of YFP-p38SJ expression in U-87MG and a reduced level of AKT, which controls several signaling pathways involved in cell proliferation and other proteins involved in cell cycle progression (Fig. 4, right panel).

Next, we evaluated the effect of p38SJ on cell cycle progression. In this study, first we synchronized U-87MG and NIH 3T3 cells by serum starvation and then treated the cells with p38SJ extract. Both cell lines demonstrated a noticeable delay in progression from G0/G1 to S phase after 52 h post-treatment with p38SJ (Fig. 5). At 16 h post treatment, 27% of U-87MG cells had progressed to S phase in the control samples while treated cells showed only 21% in S phase (Fig. 5a). NIH 3T3 cells displayed similar results with 37% of controls in S phase at 16 h and 5% of treated cells (Fig. 5b). Overall, the 70% of NIH 3T3 cells treated with p38SJ protein were in G0/G1 at 52 h post-synchronization, while 48% of untreated cells had reached G0/G1 by 16 h post-synchronization.

p38SJ interferes with signaling pathways which control cell cycle and survival of tumor cells

In the final series of experiments we used MAP kinase array to investigate the effect of p38SJ on the status of several kinases that are involved in the control of cell growth. As seen in Fig. 6, results from phospho-MAPK array experiments showed a reduction (more than two-fold) in phosphorylated levels of JNK, JNK Pan, RSK1 and AK2, which were observed after treatment of T98G with p38SJ (Fig. 6). Several downstream targets in the AKT2 pathway were also affected by p38SJ including NF-κB p65 (Fig. 6c, top panel). Several other proteins associated with cell growth such as E2F-1 (see Fig. 4), and several other previously shown proteins including STAT and phospho- CREB [15, 30] were influenced by p38SJ.

To further validate the role of p38SJ in the regulation of JNK signaling pathway in T98G glioblastoma, we monitored JNK activity by examining several of its downstream targets, including the transcription factor c-Jun and NF- κ B/p65. Our results showed significant reductions in the downstream phosphorylated forms of c-Jun and the level of p65, both of which are involved in cell proliferation (Fig. 6c, d).

Discussion

In this study we demonstrated that p38SJ inhibits growth of glioblastoma by interfering with events associated with control of the cell cycle. Progression of cells through the various stages of the cell cycle is mediated by a group of cyclins and their associated kinases. For example, cyclin E and its associated kinases, cdk2 and cdk6 are among the key regulators of G1 to S transition and cdc2 plays a role in G1 and G2 to M progression [31–34].

Downregulation of these proteins by p38SJ may result in reduced proliferative capacity of malignant glioma cells shown in [1] and [4]. Our results also showed that the levels of E2F1 transcription factor and PCNA which are markers of cell cycle progression and S phase, respectively, are reduced upon treatment of the glioma cells with p38SJ. The observed level of cdk9 in p38SJ treated cells is of interest as cdk9, by interacting with cyclin T1, forms a complex known as pTEF, which stimulates the elongation process of RNA polymerase II, and thus may have a general effect on overall cellular gene expression [35].

Cdk9 and cyclin T1 have also been associated with tumor transformation, cell proliferation, and anti-apoptosis in a variety of cancers, including prostate cancer, lymphomas, PNETs, and neuroblastomas [36]. Recent studies on the effect of p53 on G1 arrest have shown downregulation of Cdk9 and other complexes associated with P-TEFb. These changes may allow accumulation of polyadenylated histone transcripts that promote greater genomic stability [37]. The mechanism by which p38SJ reduces Cdk9 in glioma cells remains unknown at present, although one may speculate a role for p53 in this event. On the other hand, E2F-1 downregulation due to G1-arrest is known to reduce NPAT. NPAT, together with Cdc2, normally recruits Cdk9 in joining with RNA polymerase II. As p38SJ induces G0/G1 arrest (Fig. 5) and reductions in E2F-1 level (Fig. 4), it is possible that p38SJ, via this reduction of E2F-1, also dysregulates Cdk9 production (Fig. 4). As mentioned above, we noted that several downstream targets of cell cycle modulating proteins are affected by p38SJ [38–40].

Our results show the effect of p38SJ on regulation of several kinases that are involved in cell growth and progression through the cell cycle. The AKT family of proteins, particularly AKT2 and AKT3, has been shown to be strongly upregulated in a variety of cancers and provides stimulus for tumor cells to progress through G0/G1 and avoid induction of apoptosis via inhibition of the proapoptotic BAD protein [41]. Robust activation of AKT2 has been noted in many glioblastoma multiforme tumors, and its upregulation may provide one pathway by which anaplastic gliomas transform into grade IV gliomas. The AKT2 isoform has also been associated with increased glioma invasiveness and higher WHO grading [42]. Likewise, the ERK and JNK family of proteins have also been associated with glioma tumor malignancy, activation of EGFr, anchorage independent growth, and cell survival. Downregulation of ERK in glioma cells has been shown to delay growth and arrest

cell cycle progression at G0/G1 [43]. Mitogenic stimulation of the Ras/ERK pathway leads to the activation of Rsk1, which may also promote survival through the activation of the transcription factor NF- κ B [44]. Interestingly, inhibition of AKT2 induces autophagy through cell cycle arrest, and downregulates p70S6K [45]. In tumors activation of the JNK MAPK was demonstrated, and overexpression of phospho JNK (Thr183/Tyr185) was found [46]. Inhibition of JNK phosphorylation results in growth arrest and cell cycle deregulation, which suggest constitutive JNK activity is necessary to promote proliferation in tumors [46]. Interestingly, while JNK signaling and c-Jun activation is important for cell proliferation in tumors, it also leads to neuronal death via induction of apoptotic signals [47].

In summary, we have demonstrated that p38SJ protein strongly inhibits glioma cell proliferation (Figs. 1, 2, 3) via the arrest of cell cycle progression at G0/G1 (Fig. 5). This effect is accompanied by the downregulation of several phosphate-modulated proteins involved in cell proliferation and anti-apoptotic events (Figs. 4, 6). These data suggest that p38SJ may be a promising therapeutic in the treatment of malignant gliomas, either as an antiproliferative drug or as a radiosensitizer.

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Fig. 1. p38SJ inhibits cell proliferation. **a** Cell viability assay using T98G glioma cells transfected with various plasmids expressing p38SJ or their control empty vectors. The quantitative representation of cell viability assay is displayed after 5 days. **b** Colony formation assay showing representative images of T98G glioma cells with and without p38SJ-expressing plasmids (*top panel*). The quantitative representation of colony assays for T98G and U-87MG glioma cells is displayed (*bottom panel*). The number of colonies that survived in the presence of blasticidin was determined after 3 weeks as described in materials and

methods. The average number of colonies from three independent experiments were determined and arbitrarily set at 100% for the control

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Fig. 2.

N-terminal region of p38SJ is important for the inhibition of cell proliferation. **a** Schematic presentation of p38SJ protein and its N-terminal deletion mutants highlighting the various regions of the protein with potential phosphate binding sites spanning between amino acids 8–149 as depicted by P (9–11), P (34), P (64), and P (143,148,149). The position of DING spanning amino acids 1–8 is shown. **b.** Colony formation assay using U-87MG cells and various deletion mutants of p38SJ. The percent inhibition of colony formation caused upon expression of p38SJ or its mutant variants is shown

Fig. 3. Effect of exogenously added p38SJ on glioma cell viability. **a** Coomassie blue stained gel illustrating purified p38SJ before and after treatment with proteinase K (PK). *M* indicates size marker proteins. **b** MTT assay illustrating percent of affected U-87MG or T98G cells after treatment with p38SJ or p38SJ plus PK

Fig. 4.
Effect of p38SJ on various proteins associated with the cell. Western blot analysis of extracts from T98G cells after treatment with p38SJ (*Panels a and b*) or U-87MG cells after transfection with YFP-p38SJ (*Panel c*)

Fig. 5.

Effect of p38SJ on cell cycle progression. **a** FACS cell cycle analysis of NIH 3T3 cells treated with p38SJ extract demonstrates a delay in entry between G0/G1 and S phases. The labeled bars at the bases of the graphs indicate the relative stage of cell cycle arrest for p38SJ treated cells compared to control cells treated with p38SJ + PK. **b** FACS cell cycle analysis of U87MG cells treated with p38SJ extract demonstrates a slow progression of cells from G0/G1 to S phase

Fig. 6.

Effect of p38SJ on MAPK signaling. **a** Phospho-MAPK arrays treated with transfected glioma cell lysates. The top panel displays results from protein extracts from T98G cells expressing only YFP, and the bottom panel displays results from T98G cells expressing YFP-p38SJ. **b** Quantitative representation of phospho-MAPK protein levels in p38SJ-expressing T98G cells as determined by densitometric analysis of each spot. The values were normalized to the levels seen in YFP expressing cells. **c** Western blot analysis of extracts from YFP-p38SJ-expressing T98G cells for NF-κB p65 and phospho-c-Jun, c-Jun, YFP-p38SJ and the housekeeping protein, Grb2. **d** Quantification of the differences in NF-κB levels as determined by densitometric analysis of the band corresponding to NF-κB p65 that was normalized to the level of Grb2 is shown. **e** Quantification of the differences in phospho-c-Jun after being normalized to the level of total c-Jun is shown. Grb2 served as a loading control